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TRANSPLANTATION OF SPERMATOGONIA ISOLATED FROM GIANT GOURAMI COLD PRESERVED TESTIS INTO NILE TILAPIA LARVAE

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ABSTRACT. The recent study has been conducted to develop testicular germ cell (TGC) transplantation as a tool for preservation and propagation of male germ-plasm from endangered fish species. In practice of TGC transplantation, recipient and donor cell may not be immediately available at the same time whereas the testis can not be survive longer when it is outside of the body. Therefore, preservation of testis tissue may be required before transplantation. The research was conducted to evaluate 1) the viability of spermatogonia isolated from short term preserved testisand 2) colonization efficiency of preserved donor cells after transplantation.Testis was preserved in physiological NaCl solution at 4^{o}C for 6, 12,24 and48 hours. Testis were dissociated in 0.5 % trypsin and 3% DNase 10 IU/µL in PBS(phosphate buffered solution) complemented with 5% FBS (fetal bovine serum), 25 mM HEPESand 1mM $CaCl_2$ to obtain testicular germ cell suspension. Testicular germ cell isolated from 24 and 48 hours preservation and labeled with PKH 26 membrane fluorescent dye then transplanted into 3 days post hatched tilapia recipient. The results showed that the viability of spermatogonia started to decrease significantly in 12 hours preservation ($P<0.05$)and in 48 hours preservation, the amount of viable cells was only 54,48±8,33%. Two months post transplantation, the efficiency of colonizationwere analyzed in recipient and the result showed insignificant difference of efficiency colonization between recipient transplanted with testicular tissue preserved 24 and 48 hours (55,56% each) and without preservation (61,11%) . In conclusion, preserved testicular tissue at 4^{o}C could be used as the source of donor cell for testicular germ cell transplantation of giant gourami into Nile tilapia.
Key words: preservation, spermatogonia, giant gourami, viability, efficiency colonization.

INTRODUCTION

Germ cell transplantation technology has been successfully performed on several species of fish (Takeuchi et al., 2004, Okutsu et al. 2006b, Lacerda et al., 2008, Majhi et al. 2009). On the application of transplantation techniques, synchronizing the availability of donor cells with recipient was being a big problem. Sometimes the cell or tissue donor is available but not ready for transplant recipients yet. In the meantime, if the donor is in the form of the testis after testicular tissue removed from the body of the fish will be at risk of damage if not immediately processed. Therefore, Itis required storage techniques

(preservation) to avoid damage to the cells prior to transplantation of gametes in the testes and to increase the survival rate of gametes as well.

There are two kinds of techniques that preserve long-term storage at temperatures below 0 ° C storage and preservation of short-term storage temperatures above 0 ° C (Browne et al. 2001). At some higher vertebrates, cryopreservation of testicular with uneven maturity level sometimes decrease cell viability, especially for cell spermatogonia or PGC (Jahnukainen et al., 2006, Ehmcke&Schlatt 2008). Cryopreservation of rainbow trout testicular germ cells produced the highest cell viability of approximately 50%, 40% less than control or without cryopreservation (Kobayashi et al. 2007). The effects caused by the cryopreservation technique is said to be inefficient for short-term storage (Jahnukainen et al. 2006). In this reasearch, we tried to perform short cold preservation of giant gouramy testes as a donor at 4 ° C. This type of preservation had not been done in any fish at all.In vertebrate animals, Eriani et al. (2008) performed preservation of ductusdeferens and the epididymis cats at 4 ° C, and resulted the male gamete cells could still be alive for up to 6 days. Perhaps, this short cold preservation in physiological solution at 4 ° C could save the germ plasm during transportation as well.

MATERIALS AND METHODS

Preservation of Testes

Five pairs of testes isolated from carp-sized adult male 700-800 g. Each testicle put in physiological NaCl solution 0.7% sterile petri dish and preserved at 4 ° C with each storage period 0, 6, 12, 24 and 48 hours. Physiological solution of NaCl 0.7% was previously given antibiotics gentamycin 1.25 µL / mL. After the storage period was completed, the next testes removed from the cooler. As much as ± 20 mg ofeach testes was dissociated in 0.5 % trypsin and 3% DNase 10 IU/µL in PBS(phosphate buffered solution) complemented with 5% FBS (fetal bovine serum), 25 mM HEPESand 1mM $CaCl_2$. Cell suspensions were washed with PBS 2 timesto remove trypsin activity. Parameters measured were spermatogonia viability. A total of 10 µL of 1 mL cell suspension dissociation yield colored with 0.4% trypan blue (1:1). Cells that die will stain with trypan blue so it looks blue, while the living will still look transparent. The total number of spermatogonia and spermatogonia number of dead counted using a hemocytometer under a microscope. Each treatment was repeated three times.The highest average ofspermatogonia viability was used as the source of donor for germ cell transplantation.

Testicular Germ Cell Transplantation

a. Preparation of donor cells.

Testicular germ cell suspension of preserved testes with high viability ofspermatogonia used as a source of donor for germ cell transplantation.After the cell suspensions were washed with PBS 2 times, the number of cells were counted using a hemocytometer under a microscope CX10 (Olympus) to determine the volume of dye or label PKH 26 is used.For visualization of colonized cells in the recipient, the donor cells labeled with the fluorescent membrane dye PKH 26

according to Sigma-Aldrich Inc. protocol. Germ cell suspension compressed to a concentration of 20,000 cells / 0.5 mL and stored at cold temperatures and without light until used.

b. Transplantation of donor cells into the genital cavity of larvae recipients

Twenty recipient of 3 days post hatching (dph) larvae were used in this research. About 0.5 mL of testicular suspension with testicular cell count of 20,000 cells were injected into peritoneal cavity of the larvae using micromanipulator (Olympus). The tilapia larvae post transplantationwere reared in aquarium (60x60x60) cm^3 until they were ready to be analyzed. To evaluate the incorporation of donor cells in recipient gonads, 2 months pt recipient observed under a fluorescence microscope Nikon E600 elliptical. Six recipients were examined each treatment. Colonization efficiency of transplantation was calculated from the percentage ratio of the number of recipients carryingspermatogonialabeled PKH26 and the total number of recipients examined.

Data Analysis

All qualitative data are presented descriptively, whereas quantitative data in the form of the value being tested statistically using ANOVA (analysis of variance), followed by Duncan's multiple range test trials to determine the significant difference between treatments. Analysis using SPSS 17.0 for Windows and MS Office Excel 2007. The difference in morphological characters tested descriptively.

RESULTS AND DISCUSSION

Spermatogonia Viability of TheGiant Gouramy Testis After Preservation

The viability of spermatogonia from giant gouramy testes after preservation were shown in Table 1. The result showed that cold preservation (4 ° C) of testicular tissue (testes) in physiological saline solution affectedspermatogoniaviability significantly ($P < 0.05$). The viability started to decrease until below 80% at storage time periode12 hours. The viability dropped dramaticallyat storage time periode 48 hours. After 48 hours preservation, nearly half of spermatogonia underwent cell death characterized by blue cells (Figure 1).

Preservationmaintain and keep the material from any damage. Preservation can be either storage at low temperatures using chemicals. Preservation in the form of reduced temperature above freezing temperature and below body temperature can decrease metabolic activity, the need for oxygen, energy consumption, and hence it can prolong the preservation of cell viability (Honaramooz& Yang 2011). However, if the cooling is done too long it will destroy the balance and cellular homeostasis that undergo cell death. Generally, short-term storage temperature is the temperature of 4 ^oC refrigerator.

Table 1 Number and viability of spermatogonia of giant gouramy testes at different period of cold preservation

Preservation periods (hours)	The average no. of spermatogonia/mg testes	The spermatogoniaviability (%)
0	31,407 ± 8,668	96.77 ± 3.23[a]
6	43,152 ± 2,240	88.37 ± 3.79[a]
12	30,504 ± 1,997	77.70 ± 3.01[b]
24	11,365 ± 3,201	74.30 ± 5.41[b]
48	19,755 ± 12,102	54.48 ± 8.33[c]

Values on in each column with same superscripts are not significantly different (P>0.05).

The imbalance in the cell is also influenced by the role of reactive oxygen species (ROS), namely oxidative agents in the results of the derived category of free radicals of oxygen metabolism during the process of cellular respiration takes place (Sikka 1996). The product is in the form of compounds ROS free radicals such as O2-, H2O2, OH-can reduce cell viability (Aitken & Baker, 2006). During the process of preservation of organ / tissue / cell metabolism continue to live with the oxidation process. If the product of ROS in cells in uncontrolled conditions, It will cause a negative effect on the cells.

Analysis of Colonization Efficiency of Giant GouramySpermatogonia Isolated from The Preserved Testesin Tilapia Gonad

The results of the identification of donor cells in2 months ptrecipient gonads showed that both cell spermatogonia from the testes preserved in NaClfor 24 hours or 48 hours were able to migrate and colonized in the gonads recipient. The average of colonization efficiency of spermatogonia from the preserved testis were slightly lower than without preservation, but from a statistical test indicated that the efficiency of colonization both spermatogonia cells derived from testicular preservation and without preservation showed no significant difference (P> 0.05) (Figure 2).

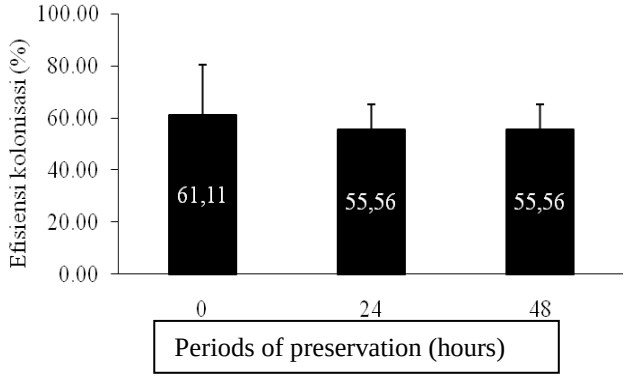

Figure 2 Colonization efficiency of giant gouramyspermatogonia isolated from the testes which preserved in NaCl solution at 4^{o}C for 0, 24 and 48 hours in the recipient tilapia.

This suggests that cold preservation at 4 ° C did not eliminate the spermatogonia cell response to chemoattractant released by the microenvironment, including somatic cells in the gonad area so that recipients carp spermatogonia cells are still able to migrate to the gonads channel tilapia larvae.

Observation on the fluorescence microscope also showed that cells isolated spermatogonia from the testes were preserved not only colonized the recipient male gonads (testes) but also on the female gonads or ovaries (Fig. 3). This suggests that the process of testicular preservation at 4 ° C for up to 48 hours does not eliminate the ability of development plasticity of cell spermatogonia that carp carpspermatogonia cells that colonized can develop into male and female germ cells during niche of the recipient to support these developments. Gender differentiation is more influenced by the somatic cells of the gonadal tissue than the control of exogenous cell itself (Yoshizaki et al., 2010).

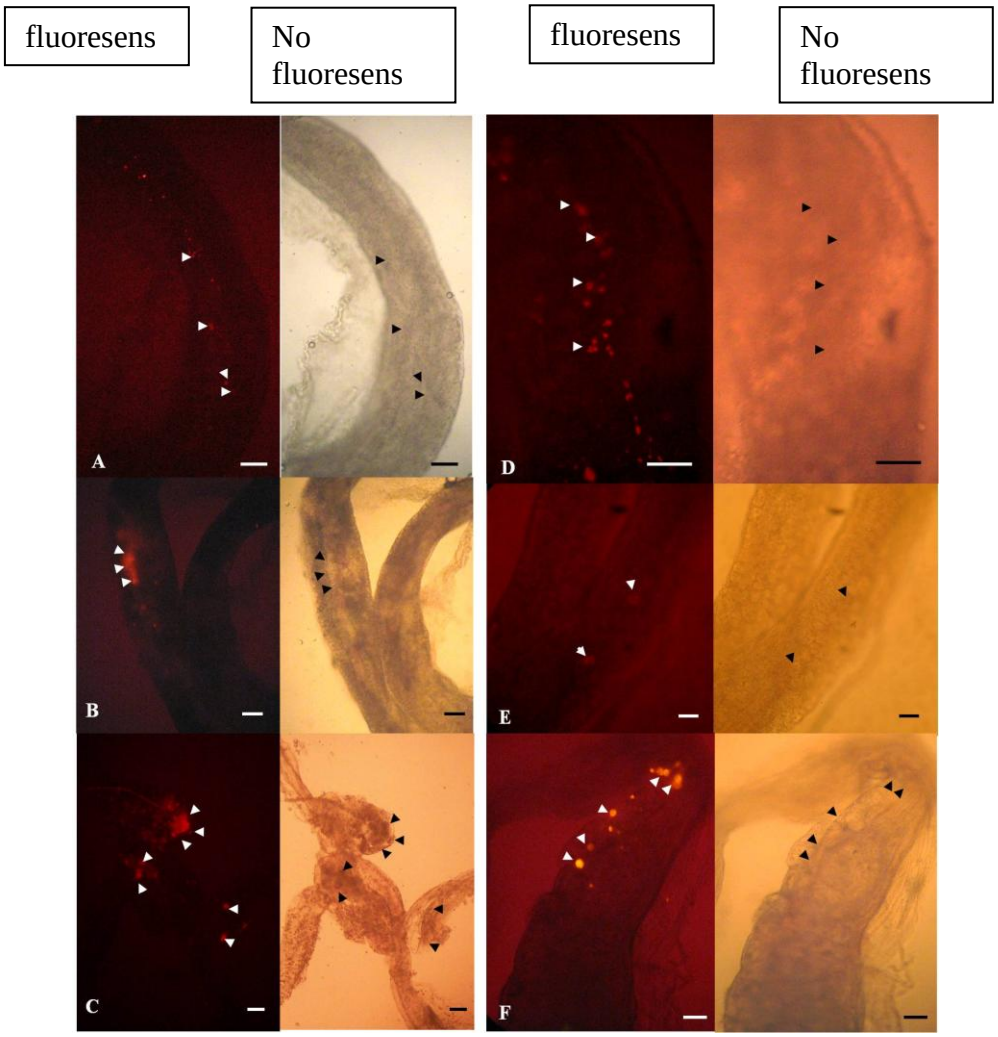

Figure 3 Recipients of tilapia gonads carrying donor cells from the giant gouramy testes in which preserved and without preservation. A-C: testis, D-F: ovarian, A, D: without preservation, B, E: 24 hour preservation, C, F: 48-hour preservation. The arrows indicate spermatogonia of giant gouramy colonized in the gonad of tilapia. Scale: 100 μm.

Cold preservation technique is a short-term preservation techniques. This technique is very easy to apply in the field because it only needs the cooler (cool box) and a physiological solution. Physiological solution such as NaCl, PBS can also be a medium buffer and maintain the physiological pH (7.2 to 7.6) as well as providing an ionic liquid environment for cell metabolism (Daniel, 1971). With this cold preservation technique, testicular tissue from death fish would also potentially be saved and used as a source of donor so that the issue of availability of donor cells that have been the limiting factor in transplantation activities can also be resolved. Preservation techniques can also contribute to efforts to save the fish gamete cells that are endangered may be found away from the location of the laboratory.

CONCLUSION

Testes of giant gouramy could preserved at 4 oC.. Cell viability decreased to 55% after preservation for 48 hours but still could be used as donor in giant gouramy germ cell transplantation into nile tilapia.

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